BATTLE OF THE SEXES: NEW INSIGHTS INTO GENETIC PATHWAYS OF GONADAL DEVELOPMENT

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ABSTRACT

Sex determination is governed by a series of genetic switches that influence cell fate and differentiation during critical periods of gonadal development. Remarkably, the primordial fetal gonad is bipotential. Therefore, gonadal development provides an excellent opportunity to identify genes involved in differential organogenesis. The identification of the testis-determining gene, *SRY* (Sex-reversed on the Y), was a pivotal first step towards unraveling this genetic pathway. It is now clear that numerous other genes, in addition to *SRY,* are necessary for normal testis development. For example, human mutations in a variety of genes (*SOX9, WT1, SF1*) impair testis development. Murine models provide evidence for additional genes (*Lhx9, Emx2, M33, Dmrt, Fgf9*). This lecture will highlight insights gleaned from human mutations in the nuclear receptors, SF1 (Steroidogenic Factor1) (NR5A1) and DAX1 (Dosage-sensitive sex reversal, Adrenal hypoplasia congenita, X chromosome) (NR0B1). These studies reveal the exquisite sensitivity of SF1-dependent developmental pathways to gene dosage and function in humans.

Keywords: DAX1, SF1, sex determination, testis, gonadotropins

INTRODUCTION

During early gonadal development, a "battle of the sexes" is played out, as the bipotential gonad differentiates into either testis or ovary. Gene dosage plays a critical role in gonadal determination. Remarkably, however, the mechanism for sex determination varies widely among species, and is not highly conserved across evolution (Table 1). For example, in Drosophila, the ratio of X chromosomes to autosomes determines sex, emphasizing the importance of gene dosage. In many reptiles, temperature of the environment is the major determinant of the

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TABLE 1

Mechanisms of Sex Determination Across Evolution

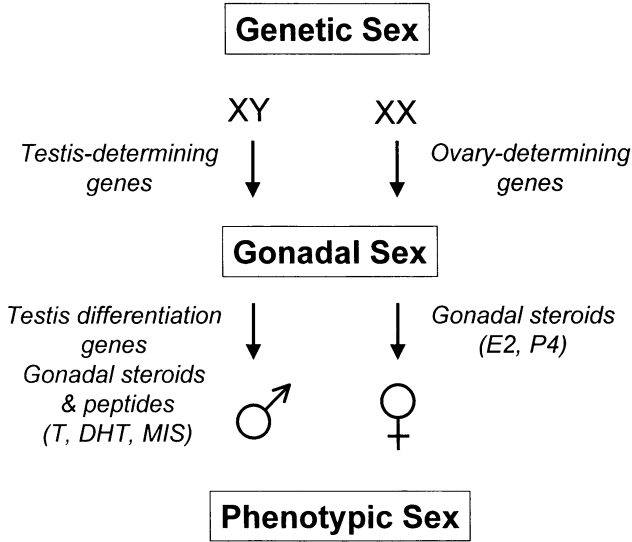| Species	Sex Determination Mechanism
Hymenoptera	Genome copy number (2x female; 1x male)
Drosophila	Ratio of X chromosomes: Autosomes
Reptiles	Temperature
Turtles	Male: cool Female: warm
Alligators	Male: warm Female: cool
Fish	Social factors; Hormonal environment
Birds	Male (ZZ) Female (ZW)
Mammals	Male (XY) Female (XX)

Sex Determination

FIG. 1. Overview of Sex Determination. T, testosterone; DHT, dihydrotestosterone; MIS, Müllerian inhibiting substance; E2, estradiol; P4, progesterone.

ratio of males to females. In birds, males are the homogametic (ZZ) sex and females are heterogametic (ZW) for sex chromosomes. Mammals, in contrast, reverse this phenomenon, as females are XX and males are XY.

Mammalian sex determination can be divided into 3 major stages: 1) genetic sex; 2) gonadal sex; and 3) phenotypic sex (Figure 1). These designations are largely conceptual, as genetic and developmental pathways link each of these phases along a continuum that ultimately

leads to a male or female capable of reproduction. Based on these stages, clinical disorders of sexual differentiation can be logically grouped into chromosomal disorders (genetic sex), dysgenetic gonads (gonadal sex), or abnormalities of hormone production or action (phenotypic sex).

Sex chromosomal composition (XX vs XY) is a critical determinant of gonadal sex. Most simplistically, this observation implies that Y-chromosomal genes are necessary for testis determination and/or the presence of two X-chromosomes favors ovarian development. Beginning with the discovery of *SRY* (Sex-reversed on the Y), much has been learned about the genes that lead to testis development; less is known about the genetic pathways that direct ovary development. In many respects, ovary development appears to be constitutive. That is, in the absence of testis-determining genes, an ovary develops, likely due to the inherent ability of germ cells to enter meiosis if an inhibitory signal is not produced by the supporting Sertoli cells. Once an ovary has formed, a number of genes are necessary to ensure oocyte survival and normal follicle maturation (e.g., *GDF9, FOXL2, FSHR*). This review will focus primarily on testis differentiation and new insights gained from naturally occurring human mutations in the nuclear receptors, SF1 (steroidogenic factor1) and DAX1 (Dosage-sensitive sex reversal, Adrenal hypoplasia congenita, X chromosome, gene 1).

Testis determination requires a series of developmental "switches" that direct the differentiation of Sertoli and Leydig cells from progenitor cells in the bipotential gonad (or urogenital ridge) (Figure 2). These biological events are initiated by a transient wave of SRY (sex-related gene on the Y chromosome) expression that alters the fate of cells in the undifferentiated gonad to give rise to Sertoli instead of granulosa cells (1). Once Sertoli cells form, they coalesce with peritubular myoid cells that migrate in from the mesonephros to form testicular cords, the progenitor to the seminiferous tubules. Sertoli cells also produce MIS (Müllerian inhibiting substance, also known as Anti-Müllerian Hormone, AMH) and inhibin. MIS causes the regression of Müllerian structures, precluding formation of the fallopian tubes, uterus, and upper segment of the vagina. Inhibin selectively suppresses pituitary follicle-stimulating hormone (FSH). Leydig cells secrete testosterone, which is necessary for development of the Wolffian structures including the epididymides, vasa deferentia, and seminal vesicles. Testosterone is converted to dihydrotestosterone, a potent androgen that induces virilization of the external genitalia.

SRY is a member of the HMG (high mobility group)-box family of transcription factors, and it is likely that it interacts with other tran-
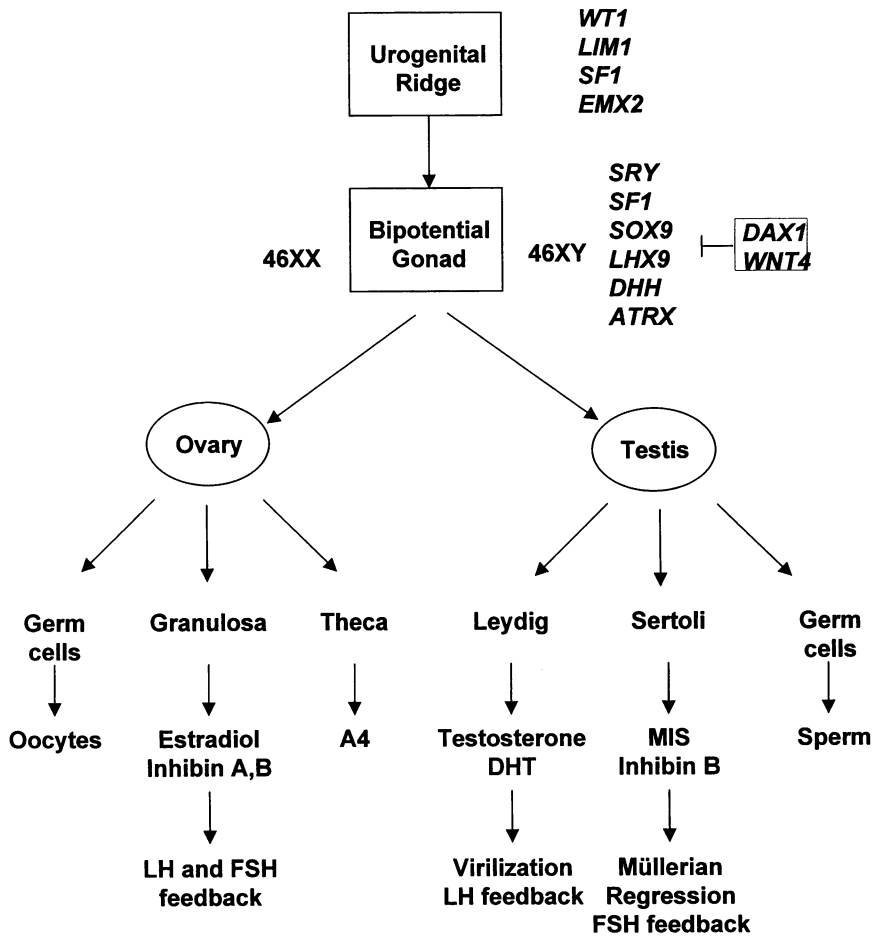
Gonadal Development

FIG. 2. Genetic Pathways Leading to Gonadal Development. The genes required for development of the urogenital ridge, bipotential and differentiated gonad are indicated, along with hormones produced by various cell lineages in the testis and ovary. A4, androstenedione.

scription factors to selectively alter the expression of target genes. However, the mechanism of SRY action remains enigmatic. A related protein, SOX9 (SRY-related HMG-box gene 9), is strikingly upregulated in the developing male gonad and is turned off in the female gonad. Targeted expression of *Sox9* is sufficient to initiate testis formation in an XX genetic female, and mutations that disrupt *SOX9*

impair testis development, indicating that it is a key gene in testis determination (2,3). Whether *SOX9* is a downstream target of SRY or is regulated independently of SRY is currently unknown. SOX9 binds to a specific site in the *Amh (Mis)* promoter, and synergizes with SF1 to regulate tissue-specific expression of MIS. SF1 is required for both adrenal and gonadal development, and it appears to function in conjunction with other transcription factors to regulate a large group of adrenal and gonadal genes. The expression pattern of DAX1 largely parallels that of SF1. In contrast to Sox9, Dax1 is down-regulated in the developing testis but not in the ovary. Overexpression of DAX1 inhibits Sry-mediated testis determination, suggesting that DAX1 may act as an "anti-testis" factor (4). The exquisite sensitivity of the male sex-determining pathways to gene dosage is apparent in humans, as haploinsufficiency of *WT1, SOX9, SF1,* and duplication of *WNT4* or *DAX1* are associated with impaired testis development in XY individuals (Table 2). In addition to those mentioned above, many other genes (e.g., *WT1, GATA4, DHH*) are also involved in gonadal differentiation and development as well as final positioning of the gonads (e.g., *INSL3, HOXA10, HOXA11*).

Mutations in *DAX1* Cause X-linked Adrenal Hypoplasia Congenita

Adrenal hypoplasia congenita (AHC) is a rare disorder characterized by adrenal insufficiency and hypogonadotropic hypogonadism (HHG). Mutations or deletions of *DAX1* (AHC, NR0B1) cause the X-linked cytomegalic form of AHC (MIM 300200) (5). Boys with this condition typically present with primary adrenal failure in infancy or childhood. HHG is manifest as delayed or absent puberty, and is caused by decreased GnRH production and impaired gonadotropin responses to residual GnRH. Infertility is caused by gonadotropin deficiency in combination with a primary testicular defect in spermatogenesis (6,7).

TABLE 2
Examples of Gene Dosage-Sensitivity of Gonadal Determination in XY Humans

Mutation	Clinical Disorder
WT1 haploinsufficiency	Wilm's tumor; ambiguous genitalia
SOX9 haploinsufficiency	Campomelic dysplasia; ambiguous genitalia
DAX1 duplication	Ambiguous genitalia
WNT4 duplication	Ambiguous genitalia
SF1 haploinsufficiency	
Heterozygous severe mutation (G35E)	Male to female sex reversal
Homozygous mild mutation (R92Q)	Male to female sex reversal

The *DAX1* gene encodes a 470 amino acid transcription factor with a carboxy-terminal region that resembles the ligand binding domain (LBD) of nuclear receptors (Figure 3). The carboxyterminus of DAX1 confers potent transcriptional silencing activity (8,9). In contrast to other nuclear receptors, the amino-terminal half of DAX1 consists of a
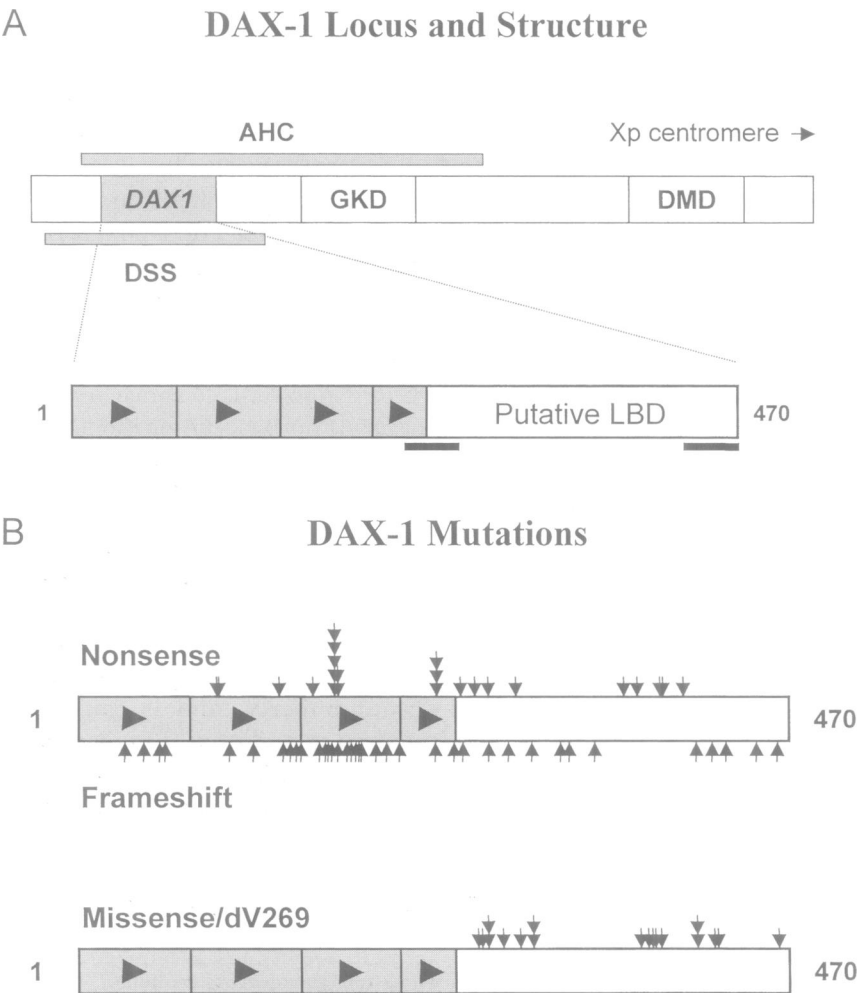

FIG. 3. Structure of DAX1 and Locations of Mutations. (A) The DAX1 locus on the X chromosome is indicated. The carboxyterminus of DAX1 is structurally related to nuclear receptors and possesses potent repressor activity. The aminoterminus contains three and one-half repeats of a 66–67 amino acid repeat motif. (B) Locations of nonsense, frameshift, and missense mutations. Note that missense mutations cluster within the carboxyterminus.

repeated amino acid sequence that contains LXXLL-like motifs implicated in protein-protein interactions (10).

Although loss of DAX1 function is associated with adrenal failure and HHG in humans, transient gene expression studies suggest that DAX1 is a repressor of transcription mediated by SF1, a key factor involved in adrenal and gonadal development. DAX1 repression involves direct protein-protein interactions, accompanied by corepressor recruitment (10–12). The interaction between DAX1 and SF1 appears to be mediated by the amino-terminal half of the DAX1 protein (8), and likely involves the LXXLL repeat motifs. This paradox of DAX1 action—it is necessary for adrenal development, but inhibits SF1 action—is incompletely understood at present. It seems likely that DAX1 serves distinct developmental and regulatory roles by acting selectively on various target genes, or by changing its level of expression at different stages of development.

Targeted mutagenesis of *Dax1* was used to produce a murine model of X-linked AHC (13). A "Cre-loxP" targeting strategy was employed because mutations in *Dax1* cause infertility in males; this approach allowed Cre-mediated excision of *Dax1* in the progeny of females carrying the floxed *Dax1* allele. Male *Dax1* knockout (KO) mice are hypogonadal and infertile. Testis histology in the *Dax1* KO mice reveals progressive seminiferous tubule degeneration, loss of germ cells, impaired spermatogenesis, and Leydig cell hyperplasia. The efferent ductules and rete testis are blocked by aberrantly located Sertoli and Leydig cells, creating an obstructive pathology that leads to sperm necrosis within the seminiferous tubules (14). Testicular biopsy findings in patients with AHC are similar to those of the animal model (7).

Over 80 different human *DAX1* mutations have been described (15), most of which are nonsense or frameshift mutations that cause premature truncation of the protein (Figure 3). Deletion of as few as the last nine amino acids of DAX1, which constitute a putative AF2 domain, is associated with a severe clinical phenotype (16). Relatively few missense mutations have been reported in *DAX1*. These mutations appear to cluster within restricted domains of the carboxy-terminus of the protein, potentially providing insight into important functional domains (17,18). Most *DAX1* mutations result in similar loss of transcriptional repression in functional assays, despite somewhat variable clinical presentations. This observation suggests that modifier genes or environmental factors account for variability in clinical presentation of AHC. Rarely, patients have been identified with relatively mild *DAX1* missense mutations that result in partial loss of transcriptional repression (19,20). These individuals first presented in adulthood

(rather than childhood) with evidence of mild adrenal failure or partial HHG. However, the absence of *DAX1* mutations in a relatively large group of patients with familial and sporadic forms of HHG and delayed puberty indicates that mutations are infrequent in such patients unless there is associated adrenal failure (21).

Mutations in *SF1* (Steroidogenic factor-1) Cause Adrenal Insufficiency and XY Sex Reversal

The *SF1* gene (also known as *FTZF1*) contains seven exons and has been mapped to human chromosome 9q33 (22,23). The gene encodes a 461 amino acid protein that is structurally similar to other members of the nuclear receptor superfamily (Figure 4). Identified functional domains of SF1 include a two zinc finger DBD, an A-box (or FTZF1 box), a hinge region, and an AF2 domain. The first zinc finger of SF1 contains a proximal box (P-box), which confers specificity in the recognition of DNA binding sites (24,25). The A-box appears to stabilize DNA binding (26,27). The AF2 domain of SF1 is involved in transcriptional activation. SF1 binds to DNA as a monomer, and recognizes DNA binding sites containing variations on a PyCA AGGTCA DNA sequence motif. Once bound to DNA, transactivation of target genes by SF1 involves the recruitment of coactivators such as steroid receptor coactivator-1 (SRC-1) (28), glucocorticoid receptor interacting protein (GRIP1) (29), CREB-binding protein (CBP)/p300 (30), or proline-rich nuclear receptor coregulatory protein (PNRC) (31).

The temporal and spatial pattern of SF1 expression is consistent with its critical role in adrenal development, steroidogenesis, and gonadal differentiation. In the mouse, Sf1 is first expressed in the urogenital ridge at embryonic day 9 (E9) (32), and subsequently in the adrenal primordium (E11) and adrenal cortical cells (E13) (33). A similar expression pattern is seen in humans (34,35). In Sertoli cells, Sf1 regulates the expression of *Amh,* which leads to regression of Müllerian structures in males (36). In Leydig cells, Sf1 regulates various enzyme genes involved in steroidogenesis and testosterone biosynthesis, allowing virilization of the male fetus.

Targeted deletion of Sf1 (FtzF1) in mice results in complete adrenal and gonadal agenesis, male-to-female sex-reversal, and persistence of Müllerian structures in males (37–40). The ventromedial hypothalamus (VMH) is also absent and there is decreased production of GnRH and gonadotropins (40,41).

A human *SF1* mutation was first identified in a patient with primary adrenal failure, XY sex-reversal, and persistent Müllerian structures

(42). This phenotypically female patient exhibited signs of primary adrenal insufficiency during the first two weeks of life. Laparotomy revealed normal Müllerian structures and streak-like gonads containing a few poorly differentiated seminiferous tubules surrounded by extensive connective tissue. Mutation analysis revealed a de novo heterozygous Gly to Glu (G35E) mutation within the P-box of the SF1 DNA-binding domain (Figure 4). Functional studies showed that this mutation did not
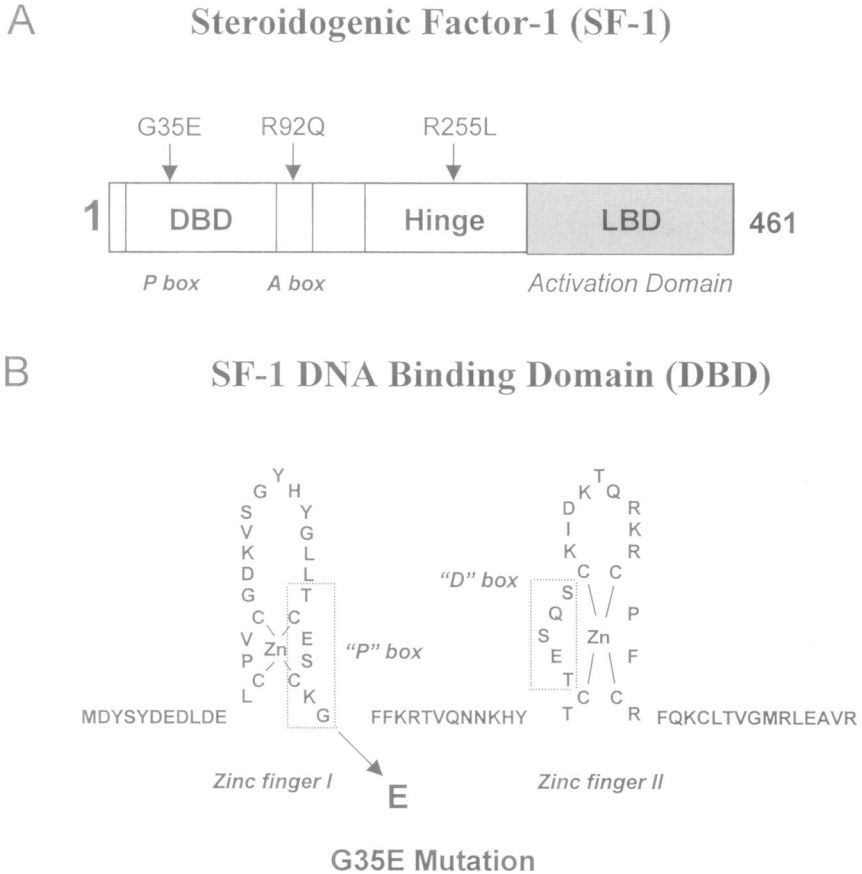

FIG. 4. Structure of SF1 and Locations of Mutations. (A) The locations of human mutations are indicated above the structure of SF-1. The positions of the mutations G35E, R92Q, and R255L within the P-box, A-box, and Hinge region, respectively, are shown. DBD, DNA-binding domain; LBD, ligand-binding domain (which consists of an activation domain). (B) Expanded view of the DNA binding domain (DBD) of SF-1, illustrating the location of the G35E mutation at the base of the first zinc finger. This mutation occurs at the position where the SF-1 DBD makes direct contact with DNA recognition sites.

interfere with protein expression or nuclear localization. However, as predicted from the location of the mutation in the DNA-binding domain, the mutant SF1 failed to bind and transactivate SF1 target genes such as *Cyp11a* (P450scc), *Dax1,* or *LHβ*. The phenotype of this patient with a heterozygous point mutation in SF1 is less severe than the complete adrenal and gonadal agenesis seen in homozygous Sf1 (−/−) knockout mice. Recent evidence suggests that heterozygous Sf1 (−/+) knockout mice also exhibit impaired adrenal function, though not as severe as that seen in this patient (43). The mutant SF1 protein does not exhibit dominant negative activity (44). Therefore, it is likely that haploinsufficiency of SF1 precludes normal glandular development and function.

The identification of additional SF1 mutations has helped to clarify the functional requirements for this receptor. A second de novo heterozygous *SF1* Arg to Leu mutation (R255L) was found in a XX female with adrenal insufficiency (45). This mutation affects a conserved residue in the hinge region of SF1. Although the mutation renders the protein transcriptionally inactive, it does not appear to impair ovarian development. A homozygous SF1 mutation was recently identified in a baby born to consanguineous parents (Figure 4) (46). This autosomal recessive mutation alters the A-box region of SF1 that modulates DNA binding by monomers (44,47). In contrast to the P-box mutation, this A-box change (R92Q) is associated with a partial loss of function and impaired binding to its response element. The observation that heterozygous family members are phenotypically normal, despite having one mutant allele, reveals the exquisite sensitivity of developmental pathways to gene dosage and residual function of SF1 in humans. Examples of the mouse and human mutations that exemplify the dose sensitivity of SF1 and DAX1 are summarized in Table 3. A striking feature of this table is that two-fold alterations in the gene doses of SF1

TABLE 3
Spectrum of SF1 and DAX1 Gene Dosage Effects on Gonadal Development

XY Gene Dose		Phenotype
SF1	DAX1	
2x	1x	Normal
1x	1x	Adrenal insuff./Dysgenetic testis
1x/0.5x	1x	Normal
0.5x/0.5x	1x	Adrenal insuff./Dysgenetic testis
0x	1x	Adrenal & Gonadal Agenesis
2x	2x (dup)	Testis dysgenesis
2x	0.5x	Late onset AHC
2x	0x	Classic AHC/Dysgenetic testis
1x	0x	Compensates for Dax1 knockout

and DAX1 yield a broad spectrum of adrenal and gonadal phenotypes. Ongoing experiments in mouse models suggest that these phenotypes are readily altered by genetic background, indicating the presence of modifier genes.

In summary, naturally occurring mutations in humans, in combination with transgenic mouse models, have provided important new insights into the mechanisms that regulate sex determination. It is increasingly apparent that the timing and levels of gene expression are critical determinants of gonadal development. Future studies should be able to further unravel this genetic pathway, providing an important paradigm for understanding cell fate determination and organogenesis.

ACKNOWLEDGMENTS

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DISCUSSION

Nathan, Boston: That really is fascinating Larry. These transcription factors, do they get expressed in other tissues, and what are the binding sites for these factors? Do they have a broader role then in gonadal development?

Jameson, Chicago: The orphan receptors SF1 and DAX1 actually have an almost identical pattern of expression in different tissues. Their expression is restricted to the gonads, adrenal gland, a portion of the hypothalamus called the ventromedial hypothalamus and the pituitary gland. Within the pituitary gland they are only present in gonadotropes cells. What's remarkable is that when there is a mutation in these factors, the clinical phenotype matches identically with the tissue specific pattern of expression. Consequently, there is adrenal insufficiency, gonadal insufficiency and hypogonadtropic hypogonadism, or LH and FSH deficiency. This is different than a lot of other factors, where there is functional redundancy. For example, when you mutate one retinoic acid receptor in a mouse, the phenotype is mild, if even observable. The same is true to some extent with thyroid hormone receptors; knockouts of these lead to a mild phenotype until you knockout the redundant TR alpha as well as TR beta form of the receptor. So it seems, at least in the case of the orphan receptors, that there is less redundancy.

The second question had to do with the binding sites. SF-1 binds to sequences that resemble many other nuclear receptors. There is a consensus motif that reads: AGGTCA. Based on x-ray crystallography, it is known that the zinc finger DNA binding domain fits into the major groove and makes contacts with the GG dinucleotide repeat within the middle. Because a lot of other receptors bind to a quite similar sequence motifs, it raises the question of how you ever get specificity associated with these sites. It seems that some of the sequences that surround consensus site have a modulating effect. It is also likely that transcription factors binding on the left and the right tend to stabilize these complexes and form very large transcriptosomes that are either permissive or not for the factors that bind. Thus, the DNA sequence is only one component of how specificity is achieved.

Marshall, Charlottesville: Larry, as always, a beautiful presentation. I was wondering about two aspects of interest. You're linking magnitude of gene expression in terms of adrenal development. In your clinical cases there are failures of adrenal function. In your mice, where you are showing differential DAX expression, can you show differential function of the adrenal? In other words, can you link dose of gene to adrenal secretory response? Secondly there are normal clinical events during maturation, for example, adrenarche for which we have no physiologic explanation. Is there any evidence that any of these genes are expressed or re-expressed to a greater degree during normal adrenal maturation?

Jameson: Those are very interesting questions. Dax 1 is an antagonist of Sf1. When we eliminate Dax1 you actually get adrenal hyperfunction. There are, of course, compensatory physiologic responses that modulate this adrenal hyperfunction. But, if you stress a Dax1 knockout mouse they tend to secrete corticosterone more exuberantly. So, in fact, the adrenal response to these transcription factors does appear to be graded or dose-related. We have now crossed our Dax1 knockout mice to Sf1 heterozygote knockout mice. In this case, the mice have only one dose of Sf1. When we cross this mouse to a Dax1 knockout, there is a rescue the phenotype of the Sf1 heterozygote so that residual Sf1 works better in the absence of the Dax1 repressor protein. The question about adrenarche remains illusive. Unfortunately, the structure of the mouse adrenal is fairly different from primates, and there really is not a comparable androgen secreting zone in the mouse adrenal.

Billings, Baton Rouge: A few years ago Maria New of New York presented a paper at

this Association which was in entitled "Pope Joan: A Recognizable Syndrome," and if I recall, there was some sort of hydroxylase deficiency that occurred which resulted in ambiguous genitalia and a Pope that may or may not have been male. Do the genetic pathways you discussed have to do with a hydroxylase deficiency resulting in someone who is genotypically female, becoming male? Dr. New presented Pope Joan in conjunction with a man who was bleeding each month and thereby explained it all. I'm not an endocrinologist; I was just wondering how it all fit together.

Jameson: I didn't hear Maria's talk, but she's the world's authority on congenital adrenal hyperplasia caused by 21 hydroxylase deficiency. In that disorder, because the production of cortisol is blocked, ACTH is markedly up-regulated, stimulating the adrenal gland to grow and become hyperplastic. Related to John Marshall's question about adrenarche, the chronic stimulation by ACTH leads to overproduction of adrenal androgens, which are increased even further because there is the enzymatic block tends to shunt all the precursor steroids into the androgen pathway. Thus, XX individuals become virilized, if not treated to suppress over production of adrenal androgens. The name of this disorder—congenital adrenal hyperplasia—is similar to the one associated with DAX1 deficiency—adrenal hypoplasia congenita. But, the latter disorder is associated with a small adrenal gland and there is no androgen excess.

Benz, Boston: I was struck in your first couple of slides about the relatively small number genes that have been found to be critical for sex differentiation.

Jameson: Ed, some of these genes, such as DAX1 encode transcriptional repressors. It is also notable that DAX1 has been shown mediate RNA shuttling between the nucleus and cytoplasm. Several repressor proteins, including some involved in C. elegans germ cell development, are known to also regulate splicing. Bert O'Malley's group has also shown that certain transcriptional coactivators, which interact with nuclear receptors, can regulate alternative splicing. To date, however, none of the mammalian sex determining genes has been shown alter RNA splicing but this remains an intriguing possibility.